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A carbon dioxide avoidance behavior is integrated with responses to ambient oxygen and food in *Caenorhabditis elegans*

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Homeostasis of internal carbon dioxide (CO_2) and oxygen (O_2) levels is fundamental to all animals. Here we examine the CO_2 response of the nematode *Caenorhabditis elegans*. This species inhabits rotting material, which typically has a broad CO_2 concentration range. We show that well fed *C. elegans* avoid CO_2 levels above 0.5%. Animals can respond to both absolute CO_2 concentrations and changes in CO_2 levels within seconds. Responses to CO_2 do not reflect avoidance of acid pH but appear to define a new sensory response. Sensation of CO_2 is promoted by the cGMP-gated ion channel subunits TAX-2 and TAX-4, but other pathways are also important. Robust CO_2 avoidance in well fed animals requires inhibition of the DAF-16 forkhead transcription factor by the insulin-like receptor DAF-2. Starvation, which activates DAF-16, strongly suppresses CO_2 avoidance. Exposure to hypoxia (<1% O_2) also suppresses CO_2 avoidance via activation of the hypoxia-inducible transcription factor HIF-1. The *npr-1 215V* allele of the naturally polymorphic neuropeptide receptor *npr-1*, besides inhibiting avoidance of high ambient O_2 in feeding *C. elegans*, also promotes avoidance of high CO_2. *C. elegans* integrates competing O_2 and CO_2 sensory inputs so that one response dominates. Food and allelic variation at NPR-1 regulate which response prevails. Our results suggest that multiple sensory inputs are coordinated by *C. elegans* to generate different coherent foraging strategies.

carbon dioxide sensing | natural variation | oxygen sensing

CO_2 is an important sensory cue for many organisms. Insects can use elevated CO_2 as part of an alarm signal or to find food (1–3). In fungi, high CO_2 can induce filamentation (4) and regulate sporulation (5). Nematode parasites of plants and animals can follow CO_2 gradients to locate their hosts (6, 7). Internal CO_2 levels also provide important signals. For example, insects and mammals monitor internal CO_2 to modulate respiratory exchange (8–10). This homeostatic function prevents respiratory poisoning and pH changes in body fluids, which can occur if CO_2 levels rise above 5% (11).

Several mechanisms have been implicated in sensing CO_2. In *Drosophila*, avoidance of high CO_2 is mediated by a pair of odorant receptors (2, 12, 13). Artificially activating neurons expressing these receptors elicits the escape response (14). Less is known about how insects monitor internal CO_2 to control opening of spiracles (15). In mammals internal CO_2 levels regulate breathing, diuresis, blood pH, and blood flow (8). In most cases the molecular sensors involved are unclear although pH changes associated with hydration of CO_2 are thought to be important. Carbonic anhydrases, which catalyze the hydration of CO_2 to produce H^+ and HCO_3^-, are widely expressed in mammals. HCO_3^- has been shown to regulate the activity of a family of adenylate cyclases that is conserved from bacteria to man (16). However, the role of these enzymes in CO_2 signaling in animals is unclear. In fungi an HCO_3^--regulated adenylate cyclase modulates development in response to elevated CO_2 (4).

Caenorhabditis elegans belongs to the Nematoda, one of the largest phyla. Little is known, at a mechanistic level, about how these animals respond to CO_2. Nematodes lack specialized respiratory structures, and gaseous exchange is thought to occur through their cuticle. Previous studies have described *C. elegans* chemotaxis to HCO_3^- but have not examined responses to gradients of CO_2 (17, 18). *C. elegans* thrives in compost, mushroom beds, and decaying fruit, where it feeds on bacteria (19, 20). Broad ranges in O_2 and CO_2 concentrations exist in such environments depending on microbial growth, temperature, aeration, and moisture, and CO_2 levels can rise to 10% (21, 22). Here we investigate how *C. elegans* responds to CO_2.

Results

***C. elegans* Avoids Elevated CO_2.** To investigate how *C. elegans* responds to CO_2, we first exposed N2 (Bristol) wild-type animals to spatial CO_2 gradients. Gas gradients were set up over worms on agar surfaces using microfluidic chambers connected to defined gas mixtures (Fig. 1 *A* and *B* and ref. 23; see *Methods*). Within these chambers laminar flow operates such that a linear gas gradient is generated by simple diffusion between the two ends of the chamber. Unless otherwise indicated, O_2 was kept at 21% in these mixtures: CO_2 was increased at the expense of N_2. When only air was pumped into the chamber, N2 animals distributed equally to both sides of the chamber space (Fig. 1*A*). However, on introduction of a 5% to 0% CO_2 gradient, animals rapidly (<10 min) vacated areas of the chamber where CO_2 levels were high (Fig. 1*B*). To examine the concentration dependence of *C. elegans* CO_2 avoidance, we also assayed animals in gradients of 0.25% to 0%, 0.5% to 0%, 1% to 0%, and 3% to 0% CO_2. Avoidance of CO_2 was concentration-dependent, and animals avoided high CO_2 both in the presence and in the absence of a lawn of *Escherichia coli* food [Fig. 1 *C* and *D* and supporting information (SI) Fig. S1]. However, bacteria slightly but significantly reduced the strength of the avoidance response (Fig. 1 *C* and *D* and Fig. S1). The significance threshold for *C. elegans* CO_2 response was 1% CO_2 on food and 0.5% CO_2 off food at the 0.01% significance level (Fig. 1 *C* and *D* and Fig. S1). Thus, CO_2 is a potent repellent for N2 animals.

To provide a simple measure for the CO_2 response we calculated a chemotaxis index by subtracting the number of animals in the low CO_2 half of the chamber from the number in the high CO_2 half and dividing by the total number of animals in the assay. Chemotaxis indices of +1, 0, and −1 indicate perfect attraction, indifference, and perfect avoidance of CO_2, respectively. The chemotaxis indices for CO_2 gradients of 1% to 0%, 3% to 0%, and 5% to 0% were

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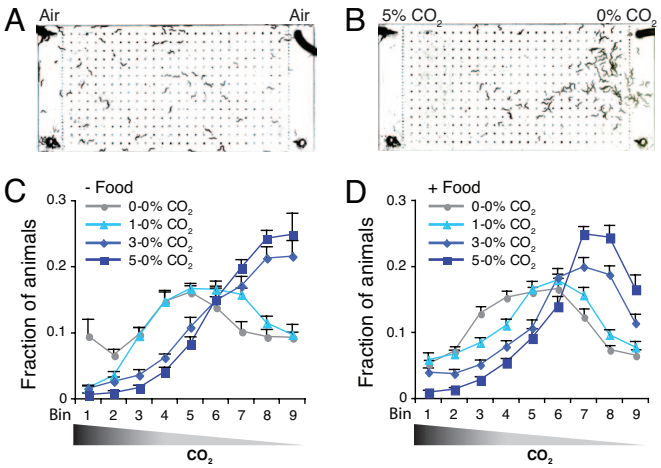

Fig. 1. *C. elegans* avoids elevated levels of CO_2. (*A* and *B*) Distribution of N2 animals in microfluidic devices after 10 min without a CO_2 gradient (*A*) or with a 5% to 0% CO_2 gradient (*B*). Assays are in the absence of food. Gases pumped into the chamber are indicated at the top. (*C* and *D*) Distribution of N2 animals in CO_2 gradients in the absence (*C* and Table S1) or presence (*D*) of *E. coli* food (see also Fig. S1). Bin numbers refer to different portions of the microfluidic chamber. High CO_2 is to the left, as indicated by the wedge. Distribution of animals in all CO_2 gradients shown was significantly different from 0–0% CO_2 ($P < 0.0001$). Distribution of animals in all CO_2 gradients shown on food was significantly different from that off food ($P < 0.0001$). In this and all subsequent figures measurements were taken 10 min after the assay began.

−0.28, −0.66, and −0.80, respectively (see Fig. S1*C*, Assays without food).

To examine how *C. elegans* avoids CO_2, we exposed N2 animals to temporal CO_2 gradients by pumping defined gas mixtures at set rates into a behavioral arena. We subjected animals to both increases (0% to 3% or 5%) and decreases (from 3% or 5% to 0%) in CO_2. Animals subjected to a 0–3% rise in CO_2 responded within 10 s of the gas switch; forward movement briefly ceased, animals reversed, and by ≈25 s after the switch most animals had committed a near to 180° turn (Movie S1, Animals on food). To quantify this response we took a reversal to be backward movement of an animal by greater than one-quarter of its body length and a turn to be when an animal brings its head close to its tail to create the shape of the Greek letter omega (Ω). Raising CO_2 levels transiently stimulated reversals and turns both in the presence and absence of bacterial food (Fig. 2 *A–D*). Reversals were sustained for longer in the presence of food, suggesting that bacterial signals modify *C. elegans* CO_2 response pathways (Fig. 2 *A–D*). Because the responses do not persist after CO_2 levels plateau at 3%, they are likely evoked by a neural circuit that responds to changes in CO_2 concentration rather than absolute concentrations.

CO_2 Stimulates *C. elegans* Locomotory Activity on Food but Not off Food. The speed an animal moves at influences how rapidly it can escape an aversive cue. This led us to examine whether elevated CO_2 stimulated movement in *C. elegans*. Raising CO_2 from 0% to 5% led to a doubling of the average speed of feeding N2 animals, from 46 to 92 μm/s (Fig. 2*E*). Unlike the increase in reversals and turns, which lasted for only 1–2 min (Fig. 2 *A–D*), the increased rate of movement was sustained as long as CO_2 levels remained high (>30 min; Fig. 2*F*). This perdurance suggests that absolute levels of CO_2, rather than change in its concentration, can signal to control speed of movement.

When returned from 5% CO_2 to 0%, feeding animals showed a further transient increase in speed before slowing down to the speed they exhibited before the CO_2 rise (Fig. 2*E*). In contrast to our observations in the presence of food, raising CO_2 levels from 0% to 5% in the absence of food caused a decrease in the average speed of movement, from 235 to 183 μm/s (Fig. 2*G*). Returning animals to atmospheric CO_2 levels reversed this inhibition. In summary, our data suggest that *C. elegans* can respond both to absolute levels of CO_2, which can regulate speed, and to changes in CO_2 levels, which modulate reversals and turns and, to some extent, speed too.

CO_2 Avoidance Is Distinct from Avoidance of Acid pH. CO_2 is potentially a complex sensory stimulus. *C. elegans* lives in aqueous films and responds to chemical stimuli dissolved in these films. CO_2 is highly soluble in water, reacting to form carbonic acid that dissociates to yield H^+ and HCO_3^- (Fig. 2*H*). HCO_3^- can dissociate further to yield H^+ and CO_3^{2-}, but CO_3^{2-} concentrations are negligible at physiological pH. Thus at the air–water interface an equilibrium is set up between gaseous CO_2 and its solvation products (Fig. 2*H*).

Previous studies have indicated that *C. elegans* avoids acid pH (24). This raised the possibility that CO_2 avoidance reflects escape from acid pH. We therefore examined how a 5% to 0% CO_2 gradient changed agar pH across the microfluidic chamber (Fig. S2). We observed a pH change of <0.1 pH units across the chamber, from pH 6.22 to pH 6.29. The small size of the pH change was expected because the agar substrate is buffered (see *Methods*). This small pH change and the previous observation that *C. elegans* avoids acid only below pH 4 (24) suggest that changes in external pH are unlikely to explain CO_2 avoidance.

C. elegans could also avoid CO_2 by responding to changes in HCO_3^- levels in the medium. To test this we examined CO_2 responses on agars buffered at different pH values, from 4.9 to 7.1. The concentrations of HCO_3^- generated by any given partial pressure of CO_2 should vary 100-fold across this pH range. We saw no substantial differences in avoidance of 5% CO_2 at different pH values (Fig. 2*I*). These data suggest that changes in external H^+ and HCO_3^- are unlikely to be the sensory stimuli that trigger CO_2 avoidance. However, the permeability of CO_2 across lipid bilayers is high (≈0.35 cm s^{-1}) (25), and the *C. elegans* genome encodes several genes with homology to carbonic anhydrases, the enzymes that catalyze hydration of CO_2 (www.wormbase.org). *C. elegans* could therefore sense CO_2 fluctuations by monitoring internal (extracellular or intracellular) H^+ or HCO_3^- levels. Alternatively, *C. elegans* could respond to molecular CO_2.

Signaling Through cGMP-Gated Ion Channels Contributes to CO_2 Avoidance. Two major chemosensory pathways have been defined in *C. elegans*. One is mediated by a cGMP-gated ion channel encoded by the *tax-2* and *tax-4* genes (26, 27). A second is mediated by transient receptor potential V-like (TRPV-like) ion channels encoded by *osm-9* and its associated subunits encoded by *ocr* genes (28, 29). We tested whether mutations in these genes disrupted CO_2 avoidance. Loss of *osm-9* did not cause a carbon dioxide avoidance defective (Cdad) phenotype in the presence or absence of food (Fig. 3*A*). In contrast, mutations in *tax-2* or *tax-4* completely disrupted CO_2 avoidance on food but only partially disrupted avoidance off food (Fig. 3*A*). Thus, cGMP pathways contribute to CO_2 avoidance, but other signal transduction pathways may also be important.

Starvation Suppresses CO_2 Avoidance. *C. elegans* thrives in decaying organic matter where microbial activity can significantly raise local CO_2 levels (21, 22). It was therefore surprising that N2 animals avoided CO_2. Studies of other nematodes, both free-living bacteriophagous species (e.g., *Panagrellus silusiae*) and plant (e.g., *Meloidogyne incognita*) and animal (e.g., *Steinernema* sp.) parasites, have reported chemoattraction not chemorepulsion to CO_2 (6, 30, 31). This led us to examine whether *C. elegans* avoidance of CO_2 is context-dependent. We began by asking whether starvation alters CO_2 avoidance. We removed N2 animals from food for 1, 3, or 5 h and then tested their responses in a 5% to 0% CO_2 gradient off food. Food deprivation suppressed CO_2 avoidance: N2 animals

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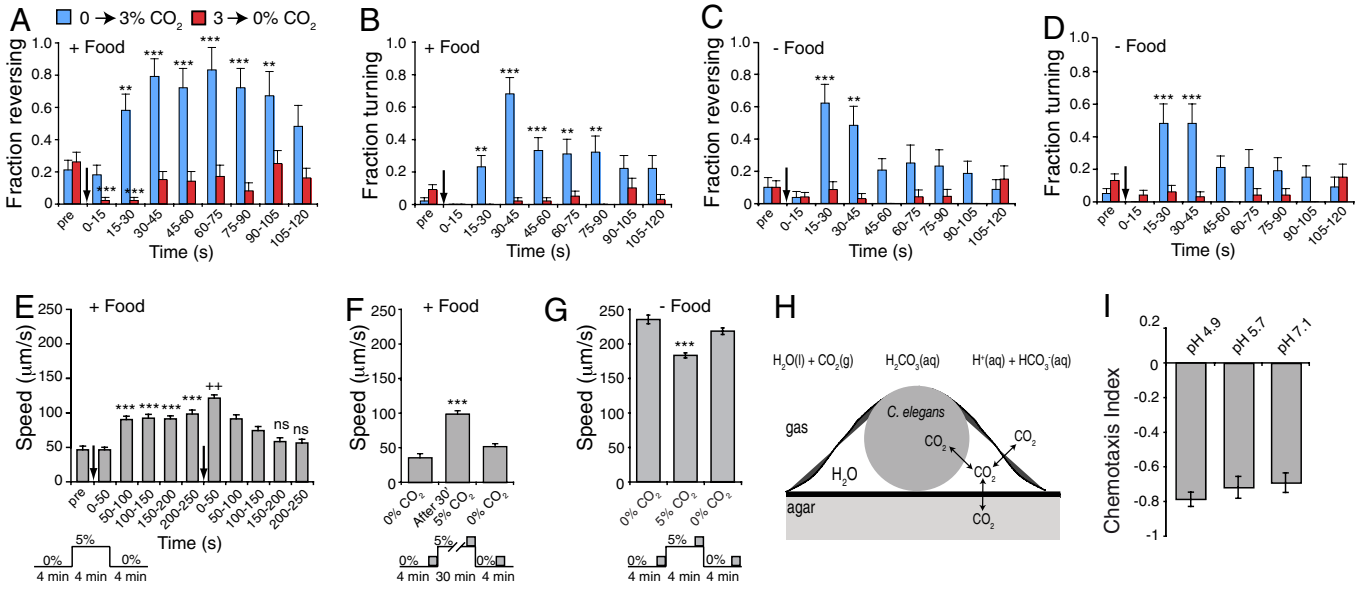

Fig. 2. Behavioral mechanisms involved in avoidance of CO_2. (*A–D*) Fraction of animals reversing (*A* and *C*) or executing a turn (*B* and *D*) after a switch in CO_2 concentration. *A* and *B* show responses on food, and *C* and *D* show responses off food. Events are binned into 15-s time intervals. Gas switches (indicated by an arrow) occur at time 0. Blue bars represent animals subjected to an increase in CO_2, from 0% to 3%; red bars represent animals subjected to a decrease in CO_2 from 3% to 0%. ''pre'' indicates responses in a 15-s interval immediately before the gas switch. Asterisks indicate significances compared with responses before the gas switch (pre). In this and all subsequent figures, *** or +++ indicates $P < 0.001$, ** or ++ indicates $P < 0.01$, and * or + indicates $P < 0.05$. (*E*) Feeding N2 animals respond to high CO_2 by increasing their movement. Animals were subjected to a rise in CO_2 (indicated by the first arrow) from 0% to 5% followed by a fall in CO_2 (indicated by the second arrow) from 5% to 0%. ''pre'' refers to speed before the first gas switch. The gas stimulus regime is indicated below the graph. Speed was measured for each animal every second and then binned into 50-s intervals. Asterisks indicate the significance compared with speed before the up step (''pre''). + indicates significance compared with the 50-s interval before the down step. (*F*) The average speed of feeding N2 animals exposed to 5% CO_2 remains elevated as long as CO_2 levels are high. Animals were exposed to 0% CO_2 for 4 min, switched to 5% CO_2 for 30 min, and then returned to 0% CO_2 for 4 min. Bars represent the average speed of animals during 50-s intervals just before increasing CO_2 levels, just before decreasing CO_2 levels, and 3 min after return of CO_2 levels to 0%. Fifty-second intervals are indicated by shaded boxes in the gas stimulus regime displayed below the graph. Asterisks indicate significance compared with speed at 0% CO_2. (*G*) In the absence of food, N2 animals respond to a rise in CO_2 by reducing their speed. Speeds were averaged over the 50-s intervals indicated by shaded boxes in the gas stimulus regime displayed below the graph. (*H*) CO_2 is potentially a complex stimulus. Aqueous CO_2 as well H^+ and HCO_3^- could be sensory cues for the nematode. Because nematodes are gas-permeable, CO_2 detection could involve both external and internal sensors. Double-headed arrows indicate equilibration of CO_2 among gas, liquid, worm, and agar phases. (*I*) Avoidance of 5% CO_2 persists with little or no change in magnitude across a broad range of external pH. All pairwise comparisons of chemotaxis indices at different pH values are not significantly different.

showed no significant CO_2 avoidance after 3 h without food and weak attraction toward CO_2 after 5 h without food (Fig. 3*B*). Thus, whereas well fed or feeding animals strongly avoid CO_2, starved animals do not.

Insulin-Like Signaling Sustains CO_2 Avoidance. Several neuroendocrine pathways signal feeding state in *C. elegans* (32–35). These include the *daf-2* insulin-like receptor pathway: high DAF-2 signaling is associated with the well fed state, whereas low signaling is associated with food deprivation. We speculated that starvation might suppress CO_2 avoidance by inhibiting DAF-2 signaling. This hypothesis predicts that mutants in this pathway would behave like starved wild-type animals even when they are well fed. Consistent with this, mutants in the insulin-like signaling pathway, including the *daf-2* insulin-like receptor, the 3-phosphoinositide-dependent kinase *pdk-1*, and the protein kinase B serine/threonine kinase *akt-1* showed reduced CO_2 avoidance or even weak attraction (Fig. 3 *C* and *D*). Insulin-like signaling thus sustains avoidance of high CO_2.

The effects of food deprivation on CO_2 responses occurred over several hours (Fig. 3*B*), a timescale consistent with a transcriptional reconfiguration of CO_2-sensing circuits. Reduced DAF-2 signaling activates the DAF-16 Forkhead transcription factor (32, 36). We therefore asked whether DAF-16 was responsible for suppressing CO_2 avoidance in *daf-2* mutants. Consistent with such a scenario, *daf-2; daf-16* double mutants strongly avoided high CO_2 and behaved indistinguishably from N2 animals (Fig. 3*C*). Together these data are consistent with a model in which starvation reconfigures CO_2 responses, at least in part, by down-regulating insulin-like signaling and activating the DAF-16 forkhead transcription factor.

Hypoxia Suppresses CO_2 Avoidance via Activation of HIF-1. Because CO_2 is the by-product of aerobic respiration, we speculated that O_2-sensing pathways might regulate CO_2 responses. One pathway regulated by O_2 is the hypoxia-inducible pathway. In both *C. elegans* and mammals, severe hypoxia (<1% O_2) induces hypoxia-inducible factor (HIF) transcription factors. In high O_2 HIFs are targeted for degradation by prolyl hydroxylases. These enzymes use molecular O_2 as a cosubstrate and are active in high, but not low, O_2. *C. elegans* encodes a single HIF, called HIF-1 (37), which is targeted for degradation by the prolyl hydroxylase EGL-9 (38). Loss of *egl-9* leads to high levels of HIF-1 irrespective of ambient O_2. *egl*-9 mutants were attracted to CO_2 (Fig. 3*E*). To investigate whether this reversal of CO_2 chemotaxis was due to high HIF-1 activity, we examined the behavior of *egl-9; hif-1* double mutants. Loss of *hif-1* restored strong CO_2 avoidance to *egl-9* mutant animals (Fig. 3*E*). Finally, we asked whether wild-type animals suppress CO_2 avoidance after experiencing hypoxia. After 1 h in 1% O_2, N2 animals, but not *hif-1* mutant animals, suppressed CO_2 avoidance (Fig. 3*F*). Taken together, these data suggest that hypoxia signals through HIF-1 to reconfigure CO_2-sensing circuits, leading to indifference or even attraction to high CO_2.

The NPR-1 Neuropeptide Receptor Promotes CO_2 Avoidance. We chose to extend our studies on the interplay between O_2 and CO_2 sensing. Previous work has shown that natural variation in the

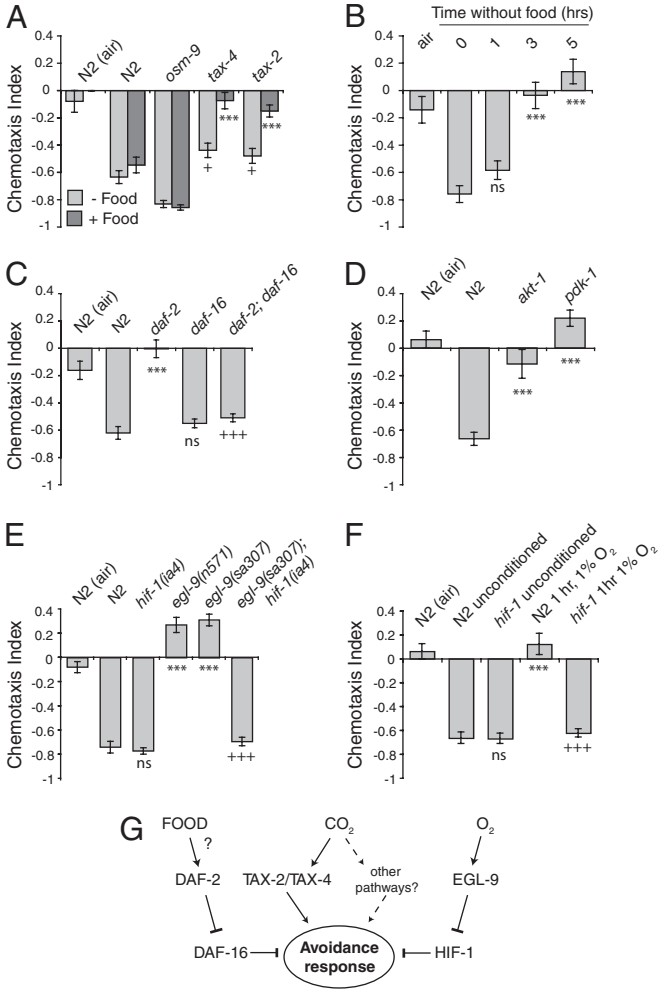

Fig. 3. Multiple signal transduction pathways contribute to CO_2 avoidance. (*A*) Disrupting the cGMP-gated ion channel encoding genes *tax-2* and *tax-4* reduces avoidance of 5% CO_2 both on and off food, whereas loss of *osm-9*, which encodes a TRPV-related channel, does not. In this and all other panels of this figure, a 5% to 0% CO_2 gradient was used to test behavior. * and + indicate significance compared with N2. Alleles used were *tax-4(p678)*, *tax-2(p691)*, and *osm-9(ky10)*. ''N2 (air)'' represents a negative control with no CO_2 gradient. (*B*) N2 animals deprived of food gradually reduce CO_2 avoidance. Asterisks indicate significance compared with unstarved N2. (*C*) Disrupting DAF-2 insulin-like receptor signaling results in strong defects in CO_2 avoidance. Reduced DAF-2 signaling inhibits CO_2 responses by activating the DAF-16 Forkhead transcription factor. Alleles used were *daf-2(e1370)* and *daf-16(mgDf47)*. Because *daf-2(e1370)* is a temperature-sensitive allele, animals were grown at 15°C and assayed at 22°C. *, significance compared with N2; ns, not significant compared with N2; +, significance compared with *daf-2*. (*D*) Mutations that disrupt *pdk-1* 3-phosphoinositide-dependent protein kinase 1 or *akt-1* protein kinase B also disrupt avoidance of CO_2. PDK-1 and AKT-1 link activation of DAF-2 to inhibition of DAF-16. Alleles used were *pdk-1(sa709)* and *akt-1(mg306)*. *, significance compared with N2. (*E*) *egl-9* mutants grown in 21% O_2 exhibit attraction to high CO_2. This switch in CO_2 response requires HIF-1. ns, not significant compared with N2; *, significance compared with N2; +, significance compared with *egl-9 (sa307)*. (*F*) Exposing feeding N2 animals to hypoxia (1% O_2) for 1 h inhibits CO_2 avoidance in a HIF-1-dependent manner. ns, not significant compared with N2; *, significance compared with N2; +, significance compared with N2 conditioned in 1% O_2 for 1 h on food. (*G*) Genetic pathways contributing to CO_2 avoidance and its modulation.

neuropeptide receptor *npr-1* alters *C. elegans* foraging behavior (39). Strains expressing the less active NPR-1 215F isoform avoid high ambient O_2 and burrow as they feed. Strains bearing the more active NPR-1 215V isoform do not avoid high O_2 as they feed and do not burrow (23, 39). We asked whether *npr-1* regulated not only

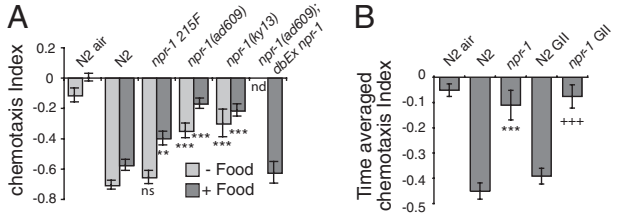

Fig. 4. The naturally polymorphic NPR-1 receptor promotes CO_2 avoidance. (*A*) Mutations in *npr-1* reduce avoidance of 5% CO_2 both in the presence and in the absence of *E. coli*; this phenotype is rescued by an *npr-1 215V* transgene. N2 animals carrying the *npr-1 215F* natural allele also exhibit reduced avoidance of CO_2 in the presence of food but maintain avoidance in its absence. ns, not significantly different compared with N2; nd, not determined. (*B*) The CO_2 avoidance defect in *npr-1* mutants is not a consequence of their aggregation behavior. *npr-1* animals grown in isolation (GII) retain a strong defect in avoidance of 5% CO_2 compared with similarly reared N2 animals. The weighted chemotaxis index was calculated by recording the position of each animal in a CO_2 gradient at 1-s intervals for 5 min and weighting this according to location in the CO_2 gradient (see *Methods*). ''N2 air'' represents a negative control with no CO_2 gradient. *, significance for comparisons between N2 and *npr-1*; +, significance between N2 GII and *npr-1* GII.

O_2 but also CO_2 responses. Consistent with this hypothesis, *npr-1* loss-of-function mutants showed striking defects in CO_2 avoidance both on and off food (Fig. 4*A*). This CO_2 avoidance defect could be rescued by an *npr-1 215V* transgene (Fig. 4*A*).

To test whether the natural *npr-1 215F* allele also modified CO_2 avoidance, we compared the behavior of N2 animals to a near isogenic strain, AX613, which bears the *npr-1 215F* allele from the German wild strain RC301 backcrossed 20 times into N2. Animals bearing *npr-1 215F* showed a significant reduction in CO_2 avoidance compared with N2 on food but not off food (Fig. 4*A*). Thus, animals having high *npr-1* activity strongly avoid CO_2 whereas animals with low *npr-1* activity exhibit weaker avoidance.

Under normal cultivation conditions *npr-1* mutant animals aggregate strongly. One explanation for their reduced CO_2 avoidance is a difference in experience compared with N2. To explore this possibility we grew N2 and *npr-1* animals in isolation. *npr-1(ad609)* animals grown in isolation retained a strong defect in CO_2 avoidance (Fig. 4*B*). Together, these data suggest that signaling from the NPR-1 neuropeptide receptor promotes CO_2 avoidance, particularly when food is present.

Sensory Integration of CO_2 and O_2 Signals in *C. elegans*. In our previous experiments we exposed animals to gradients of CO_2 in a background of 21% O_2. However, in nature *C. elegans* is likely to encounter simultaneous gradients of O_2 and CO_2. To explore how *C. elegans* navigates these more complex situations, we placed animals in combined gradients of O_2 and CO_2 with 11% O_2 and 5% CO_2 at one end of the chamber and 21% O_2 and 0% CO_2 at the other. As controls we tested animals in identical gradients of only CO_2 or O_2. Integration of CO_2 and O_2 stimuli was particularly interesting in the context of different alleles of *npr-1* because natural variation at this receptor modifies both CO_2 and O_2 responses. We therefore tested strains carrying *npr-1 215V* (which occurs in N2 and all dispersing wild isolates), *npr-1 215F* (which occurs in all aggregating wild isolates), and the loss-of-function mutant *npr-1(ad609)*.

The response of N2 animals in the crossed gradient was dominated by CO_2 avoidance: both on and off food animals accumulated at 21% O_2/0% CO_2 (Fig. 5). Thus, the avoidance of high O_2 by N2 animals when food is absent was suppressed by avoidance of high CO_2. By contrast, the response of *npr-1(ad609)* and *npr-1 215F* animals in the crossed gradient depended on context (Fig. 5 and Fig. S3). On food, the behavior of these animals was dominated by the O_2 response: animals ignored high CO_2 to accumulate at low O_2. Conversely, off food it was the response to CO_2 that dominated:

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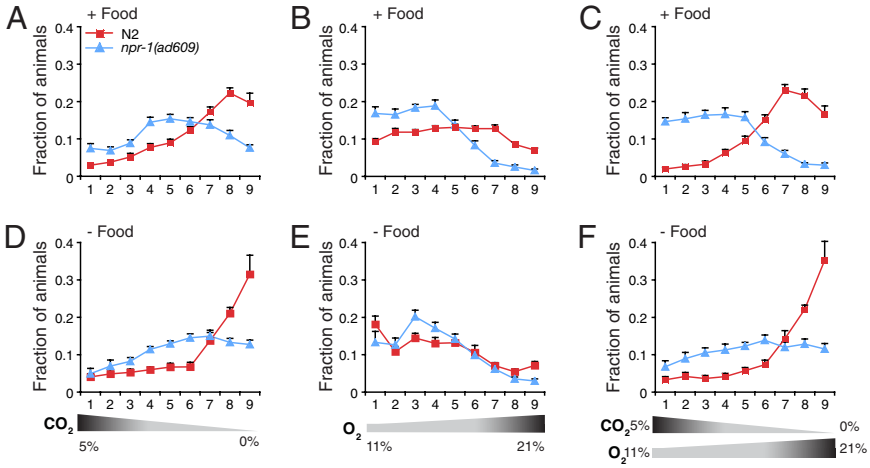

Fig. 5. *C. elegans* integrates antagonistic gradients of O_2 and CO_2 according to food availability and genotype at the *npr-1* locus. Data show distribution of N2 and *npr-1(ad609)* animals in simple and mixed gradients of O_2 and CO_2 when food is present (*A–C*) or absent (*D–F*). The gas gradients are indicated below each set of panels: 5% to 0% CO_2 in *A* and *D*; 11% to 21% O_2 in *B* and *E*; and a combined gradient of 5% to 0% CO_2 and 11–21% O_2 in *C* and *F*. N2 animals strongly avoid CO_2 both on and off food, even if this requires migration to high-O_2 environments. In contrast, the behavior of *npr-1* mutants and animals bearing the *npr-1 215F* allele (see Fig. S3) depends on context. These animals accumulate at low O_2/high CO_2 if food is present (*C*): an adverse CO_2 gradient does not appear to affect their avoidance of high O_2. Conversely, if food is absent, they tend to migrate to high O_2/low CO_2.

animals behaved as if they were in a gradient that consisted only of CO_2 (compare Fig. 5 *D–F*).

Thus, *C. elegans* integrates antagonistic inputs from CO_2- and O_2-sensing pathways to generate a coherent behavioral response in which one input dominates. The activity of the NPR-1 receptor reconfigures which of the two sensory responses dominates within the context of food availability.

Discussion

Well fed *C. elegans* avoid elevated CO_2, even though they seek environments where O_2 levels are between 11% and 7% (23, 40). The threshold we observed for CO_2 response is ≈0.5%. This is >10-fold higher than atmospheric CO_2 levels, but decaying organic matter can have much higher CO_2 concentrations, of 10% or more. *C. elegans* can respond both to absolute levels of CO_2, by modifying speed, and to change in CO_2 concentration, by altering direction of movement. Interestingly, *C. elegans* responses to O_2 are also coupled to changes in both concentration and absolute levels (40).

Behavioral and genetic dissection of the *C. elegans* CO_2 response reveals surprising complexity. Several observations are most easily explained if *C. elegans* has several pathways that respond to changes in CO_2. First, single mutations in known sensory transduction pathways are not sufficient to abolish CO_2 avoidance under all feeding conditions. Second, CO_2 responses are switched from repulsion to attraction by mutations in some genes. Third, the effects of CO_2 on speed of movement are complex. Although we have not identified CO_2-responsive sensory neurons in this study, one set of candidate neurons is those expressing the TAX-2/TAX-4 cGMP-gated ion channel.

Avoidance of CO_2 is modulated by contextual cues such as feeding state, exposure to hypoxia, and bacteria (Fig. 3*G*). Starvation completely suppresses CO_2 avoidance. This may represent a tradeoff in which food-deprived animals ignore an aversive cue to explore a wider range of environments. Previous work has shown that starvation inhibits signaling from the insulin-like receptor *daf-2* and promotes entry of the DAF-16 forkhead transcription factor into the nucleus (32). Our data are consistent with high DAF-2 signaling in well fed animals sustaining avoidance of high CO_2 and low DAF-2 signaling in starved animals reducing CO_2 avoidance by activating DAF-16. DAF-2 has been implicated in modulating behavior previously, notably in studies of salt chemotaxis and thermotaxis (33, 35, 41). The *daf-2* pathway may therefore act globally to reset behavioral state according to feeding conditions. Suppression of CO_2 avoidance in hypoxia may enable animals to migrate through CO_2-rich environments to reach more aerobic environments. Suggestions for how HIF-1 might alter CO_2 responses come from microarray studies. In both mammals and *C. elegans*, HIF regulates expression of carbonic anhydrases (42).

Bacterial signals also modulate CO_2 sensing: the CO_2 responses of well fed animals, both wild type and mutant, differ depending on whether food is present or not. Perhaps different combinations of sensory neurons mediate responses to CO_2 on and off food. Such a scenario has been described for the response of *C. elegans* to the aversive odorant octanol (43).

Sensory responses to CO_2 and O_2 are integrated by the worm in ways that depend on context and genotype at the naturally varying *npr-1* locus. Previous data have shown that NPR-1 215V suppresses avoidance of high O_2 in feeding animals. Here we show that NPR-1 215V also promotes CO_2 avoidance. By coordinately stimulating avoidance of high CO_2 and inhibiting avoidance of high O_2, *npr-1 215V* is likely to promote migration to surface environments. In contrast, the *npr-1 215F* allele permits strong avoidance of high O_2 and weak avoidance of CO_2, promoting migration to subsurface environments. We speculate that these niche preferences may favor speciation.

Why does *C. elegans* avoid CO_2? One reason may be that high external CO_2 can acidify the body fluid of *C. elegans*. However, there are other possibilities. Comparison of local O_2 and CO_2 levels may allow the animal to monitor aeration and escape from an environment before it becomes anaerobic.

In summary, *C. elegans* CO_2 avoidance defines a novel behavior. CO_2 avoidance is highly integrated with other sensory cues of natural importance to the worm, such as food and ambient O_2. One exciting challenge for the future will be to identify the neuronal substrates of CO_2 avoidance in *C. elegans* and to examine how contextual changes alter cellular behavior, leading to the alterations in organismal behavior patterns that we have observed in this study.

Methods

Strains. Strains were maintained at 22°C by using standard methods unless otherwise indicated (44). Strains used in this study are listed in *SI Materials and Methods*.

Behavioral Assays. Spatial CO_2 gradients were generated by using custom-made 33 × 15 × 0.4-mm microfluidic devices fabricated from polydimethylsiloxane

(PDMS). Design was modified from ref. 23. Devices were placed over 50–150 nematodes on nematode growth medium (NGM) agar. CO_2 gradients were formed by pumping a high percentage of CO_2 at one end of the chamber and 0% CO_2 at the other end with a syringe pump (PhD 2000; Harvard Apparatus). Flow rate through each inlet was 2 ml/min. A 5% to 0% CO_2 gradient was used in most assays; the background O_2 level was 21%. Assays were run for 10 min. The distribution of nematodes was recorded by counting animals in each of nine equal divisions of the chamber as well as in the two spaces at either end of the chamber (Fig. 1*A*). For assays in the absence of food, animals were washed with M9 Buffer before assay. Details of the wash method, which was designed to avoid giving animals a hypoxic shock, are in *SI Materials and Methods*. Assays in the presence of food were performed on NGM plates on lawns seeded 2 days earlier with OP50 (44). Defined CO_2::O_2::N_2 gas mixtures were obtained from The BOC Group.

Measurements of speed were performed by using the Digital Image Analysis System (DIAS) software as described previously (40). Each data point represents at least six assays. In all bar graphs, statistical significance was determined by using the two-tailed *t* test. In all worm distribution plots, significance was determined by pairwise comparison between different strains and conditions using Pearson's χ^2 test at the $P < 0.0001$ level. In all figures, error bars denote SEM.

Environmental Manipulations. In Fig. 2*I*, the pH of the nematode substrate was varied by using different buffers as follows: pH 4.9 (40 mM sodium acetate, pH 4.75), pH 5.7 (40 mM malate, pH 5.33), and pH 7.1 (40 mM phosphate, pH 7.2).

In starvation experiments (Fig. 3*B*), two culture plates of N2 animals were washed three times in M9 before transfer to conditioning plates (6 or 9 cm of unseeded NGM). Animals were left for 0, 1, 3, or 5 h and then washed once before being assayed off food for CO_2 avoidance.

In the hypoxia conditioning experiments (Fig. 3*F*), *C. elegans* cultures were placed in a glove box (Coy Laboratory Products) at 1% O_2 for 1 h before being assayed off food for CO_2 avoidance.

In Fig. 4*B* three animals per plate were grown from the L2/L3 larval stage to adulthood. Pools of 25 animals were then assayed in CO_2 gradients in the presence of food. The position of each worm in the PDMS chamber was recorded over a 5-min period, beginning 10 min after the onset of the assay, with a CCD camera mounted on a dissecting microscope. Resulting films were analyzed, and the positions of the worms in the chamber were determined with DIAS (Soll Technologies). See *SI Materials and Methods* for further details.

pH Measurements. We measured CO_2-induced pH changes using NGM containing 500 μM pH-sensitive chromophore 8-hydroxypyrene-1,3,6-trisulphonic acid (HPTS; Sigma). For the HPTS fluorescence (*F*) measurement method, see *SI Materials and Methods*.

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